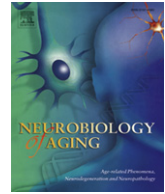




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## Neurobiology of Aging

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## Age-related changes in neuroactive steroid levels in 3xTg-AD mice

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## ABSTRACT

Although neuroactive steroids exert neuroprotective actions in different experimental models of neurodegenerative diseases, including those of Alzheimer's disease (AD), their relationships with aged related physiologic and pathologic brain changes remain to be clarified. In this study the levels of pregnenolone, dehydroepiandrosterone, progesterone, dihydroprogesterone, tetrahydroprogesterone, isopregnanolone, testosterone, dihydrotestosterone, 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol, 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol, 17 $\alpha$ -estradiol, and 17 $\beta$ -estradiol were assessed in the limbic region of young adult (7 months) and aged (24 months) male wild type and triple transgenic AD mice. Age related neuropathological changes in AD brains, such as  $\beta$ -amyloid accumulation and gliosis, were associated with modified levels of specific neuroactive steroids and particularly with changes in the levels of progesterone and testosterone metabolites. The altered levels of neuroactive steroids in aged AD brains might impact on the activation of neuroprotective signaling mediated by classic and nonclassic steroid receptors, like the gamma-aminobutyric acid (GABA)-A receptor.

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## 1. Introduction

The neuroactive steroid family includes steroid hormones produced in peripheral glands and steroids directly synthesized in the nervous system (i.e., neurosteroids) (Melcangi et al., 2008). They act as important physiological regulators of nervous function, affecting mood, behavior, reproduction, and cognition, and act as protective agents in models of injury and disease, including experimental models of Alzheimer's disease (AD), Parkinson's disease, multiple sclerosis, traumatic brain injury, stroke, autism, schizophrenia, mood disorders, and peripheral neuropathy (Melcangi et al., 2008; Panzica et al., 2012; Schumacher et al., 2012). Although neuroactive steroids have well established neuroprotective roles, their relationships with normal brain aging and age-related neurodegenerative diseases remain incompletely understood. Recent studies have shown that levels of neuroactive steroids can be affected by pathology or injury, as demonstrated in experimental models of multiple sclerosis, diabetic neuropathy,

Parkinson's disease, and trauma (Caruso et al., 2008, 2010a; Giatti et al., 2010; Meffre et al., 2007; Melcangi and Garcia-Segura, 2010; Melcangi et al., 2012; Pesaresi et al., 2010), using highly sensitive and specific analytical methods, such as liquid chromatography tandem mass spectrometry (LC-MS/MS).

Still unclear is the relationship between neuroactive steroids and AD. Evidence from a limited number of studies in postmortem human brain suggests that age-related depletion of at least some neuroactive steroids might contribute to development of AD. For example, brain levels of testosterone in men are inversely associated with AD risk (Rosario et al., 2004, 2011). Similarly, AD in women is linked to low brain levels of 17 $\beta$ -estradiol and estrone (Rosario et al., 2011; Yue et al., 2005). Further, tetrahydroprogesterone, also known as allopregnanolone, is also reported to be significantly lower in AD (Marx et al., 2006). Examination of androgens (Rosario et al., 2006, 2010), estrogens and progesterone (Carroll et al., 2007, 2010; Rosario et al., 2010; Yue et al., 2005) and allopregnanolone (Singh et al., 2011; Wang et al., 2010) in transgenic mouse models of AD has largely supported a protective role of these neuroactive hormones against the progression of the disease. In addition to age-related losses of neuroactive steroids contributing to AD pathogenesis, other evidence indicates that AD neuropathology might alter neuroactive steroid levels. For instance, neurosteroidogenesis is impaired in cell lines exposed to  $\beta$ -amyloid

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(A $\beta$ ) peptide and oxidative stress (Schaeffer et al., 2006, 2008a, 2008b). Interestingly, increased mRNA levels of neurosteroidogenic enzymes have been reported in AD brain (Luchetti et al., 2011a, 2011b), suggesting the possibility of a compensatory response to AD-related changes in neuroactive steroids. Further investigation is necessary to clarify the relationships between aging, AD, and neuroactive steroids.

In the current study, the effects of aging and AD-like neuropathology on neuroactive steroid concentrations were characterized in triple transgenic AD (3xTg-AD) mice. Specifically, we have analyzed limbic regions of young adult (7 months) and senescent (24 months) male wild type (WT) and 3xTg-AD mice for levels of several neuroactive steroids: pregnenolone (5-pregnen-3 $\beta$ -ol-20-one; PREG); dehydroepiandrosterone (DHEA); progesterone (PROG) and its metabolites dihydroprogesterone (5 $\alpha$ -pregnane-3, 20-dione; DHP), tetrahydroprogesterone (3 $\alpha$ -hydroxy-5 $\alpha$ -pregnen-20 one; THP) and isopregnanolone (3 $\beta$ -hydroxy-5 $\alpha$ -pregnen-20 one); testosterone and its metabolites dihydrotestosterone (5 $\alpha$ -androstane-17 $\beta$ -ol-3-one; DHT), 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (3 $\alpha$ -diol), 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol (3 $\beta$ -diol); and 17 $\alpha$ -estradiol (17 $\alpha$ -E) and 17 $\beta$ -estradiol (17 $\beta$ -E). Age-related development of neuropathology in 3xTg-AD mice was assessed by quantifying immunohistochemical indices of A $\beta$  accumulation and gliosis.

## 2. Methods

### 2.1. Animals

Fifteen male homozygous 3xTg-AD mice (Oddo et al., 2003) and 13 background strain, WT mice (C57BL/6/129S; The Jackson Laboratory, Bar Harbor, ME, USA) were bred and maintained at the University of Southern California (USC) vivarium facilities with food and water available ad libitum. At 7 and 24 months of age mice were anesthetized (80 mg/kg ketamine 5 mg/kg xylazine, intraperitoneally) and perfused with ice-cold saline. Brains were bisected, with 1 hemisphere immersion fixed in 4% paraformaldehyde/0.1 M phosphate buffered saline for 48 hours. The other hemisphere was dissected into limbic regions and then frozen on dry ice for neurosteroid extraction and assessment. All experimentation was approved by the USC Institutional Animal Care and Use Committee and carried out in accordance with National Institutes of Health guidelines.

### 2.2. Immunohistochemistry

Fixed hemibrains were sectioned exhaustively in the horizontal plane at 40  $\mu$ m. Every 8th section was pretreated with formic acid (99%) for 5 minutes, then immunolabeled using antibodies directed against A $\beta$  (#71-58000, 1:300 dilution, Zymed, San Francisco, CA, USA). Immunoreactivity was visualized using an avidin:biotinylated enzyme complex immunoperoxidase method (ABC Elite; Vector, Burlingame, CA, USA) as previously described (Rosario et al., 2006). For quantification of A $\beta$  immunoreactivity load, gray scale images of high magnification fields (420  $\times$  330  $\mu$ m) were digitally captured (CCD camera coupled to an Olympus Optical BX40 microscope) then filtered with a predetermined threshold using NIH Image 1.61 to create a binary image identifying positive and negative immunolabeling. Load was calculated as a percentage of total pixel area positively labeled as previously described (Rosario et al., 2006). Mean load values were averaged from 2 to 3 nonoverlapping fields from each brain region in 5 sections per animal.

Other sections were labeled using antibodies directed against ionized calcium binding adaptor molecule-1 (IBA-1; Wako Chemicals, Neuss, Germany) as a marker of microglial activation (Ito et al., 1998) and glial fibrillary acidic protein (GFAP; clone GA5;

Sigma-Aldrich, Tres Cantos, Spain) as a marker of astrocyte reactivity (Middeldorp and Hol, 2011), as previously described (Barreto et al., 2009; Ito et al., 1998). IBA-1 and GFAP immunoreactivities were quantified by volume density morphometric analysis (Weibel, 1979). Experimenters were blinded to treatment conditions during quantification.

### 2.3. Assessment of neuroactive steroids by liquid chromatography tandem mass spectrometry

PREG, PROG, DHP, THP, isopregnanolone, testosterone (T), DHT, 3 $\alpha$ -diol, 3 $\beta$ -diol, DHEA, 17 $\alpha$ -E, and 17 $\beta$ -E were purchased from Sigma Aldrich. 17,21,21,21-D<sub>4</sub>-PREG was kindly synthesized by Dr P. Ferraboschi (Dept. of Medical Chemistry, Biochemistry and Biotechnology, University of Milano, Italy); 2,2,4,6,6-17 $\alpha$ , 21,21,21-D<sub>9</sub>-PROG was obtained from Medical Isotopes, (Pelham, NH, USA); 2,4,16,16-D<sub>4</sub>-17 $\beta$ -estradiol was obtained from CDN Isotope Pointe-Claire (Quebec, Canada). SPE cartridges (Discovery DS-C18 500 mg) were from Supelco, Italy. All solvents and reagents were high-pressure liquid chromatography (HPLC) grade (Sigma Aldrich).

Samples were extracted and purified according to Caruso et al. (2008, 2010b). Briefly, samples were added with internal standards and homogenized in 3 mL of MeOH/acetic acid (99:1, vol/vol) using a tissue lyser (Qiagen). After an overnight incubation at 4 °C, samples were centrifuged at 15,300g for 5 minutes and the pellet was extracted twice with 1 mL of MeOH/acetic acid (99:1, vol/vol). The organic phases were combined and dried with a gentle stream of nitrogen in a 40 °C water bath. Samples were resuspended in 3 mL of MeOH/H<sub>2</sub>O (10:90, vol/vol) and passed through a SPE cartridge, previously activated with MeOH (5 mL) and MeOH:H<sub>2</sub>O 10:90 (vol/vol) (5 mL). Steroids were eluted in MeOH, concentrated, and transferred into auto-sampler vials before LC-MS/MS analysis. Quantitative analysis was performed on the basis of calibration curves prepared and analyzed using deuterated internal standards. Calibration curves were extracted and analyzed as described above for samples.

Positive atmospheric pressure chemical ionization experiments were performed using a linear ion trap-mass spectrometer (LTQ, ThermoElectron Co, San Jose, CA, USA) using nitrogen as sheath, auxiliary, and sweep gas, and equipped with a Surveyor LC Pump Plus and a Surveyor Autosampler Plus (ThermoElectron Co). The mass spectrometer was employed in MS/MS mode using helium as collision gas. Samples were analyzed employing the transitions as previously reported (Pesaresi et al., 2010). The LC mobile phases were (1) H<sub>2</sub>O/0.1% formic acid; and (2) methanol (MeOH)/0.1% formic acid. The gradient (flow rate 0.5 mL/min) was as follows: T0.0 70%A, T1.5 70%A, T2.0 55%A, T3.0 55%A, T35.0 36%A, T40.0 25%A, T41.0 1%A, T45.0 1%A, T45.2 70%A, and T55.0 70%A. The split valve was set at 0–6.99 minutes to waste, 6.99–43.93 minutes to source and 43.93–55 minutes to waste. The Hypersil Gold column (100  $\times$  3 mm, 3  $\mu$ m; ThermoElectron Co) was maintained at 40 °C. The injection volume was 25  $\mu$ L and the injector needle was washed with MeOH/H<sub>2</sub>O 1/1 (vol/vol). Peaks of the LC-MS/MS were evaluated using a Dell workstation by means of the software Excalibur release 2.0 SR2 (ThermoElectron Co).

### 2.4. Statistical analysis

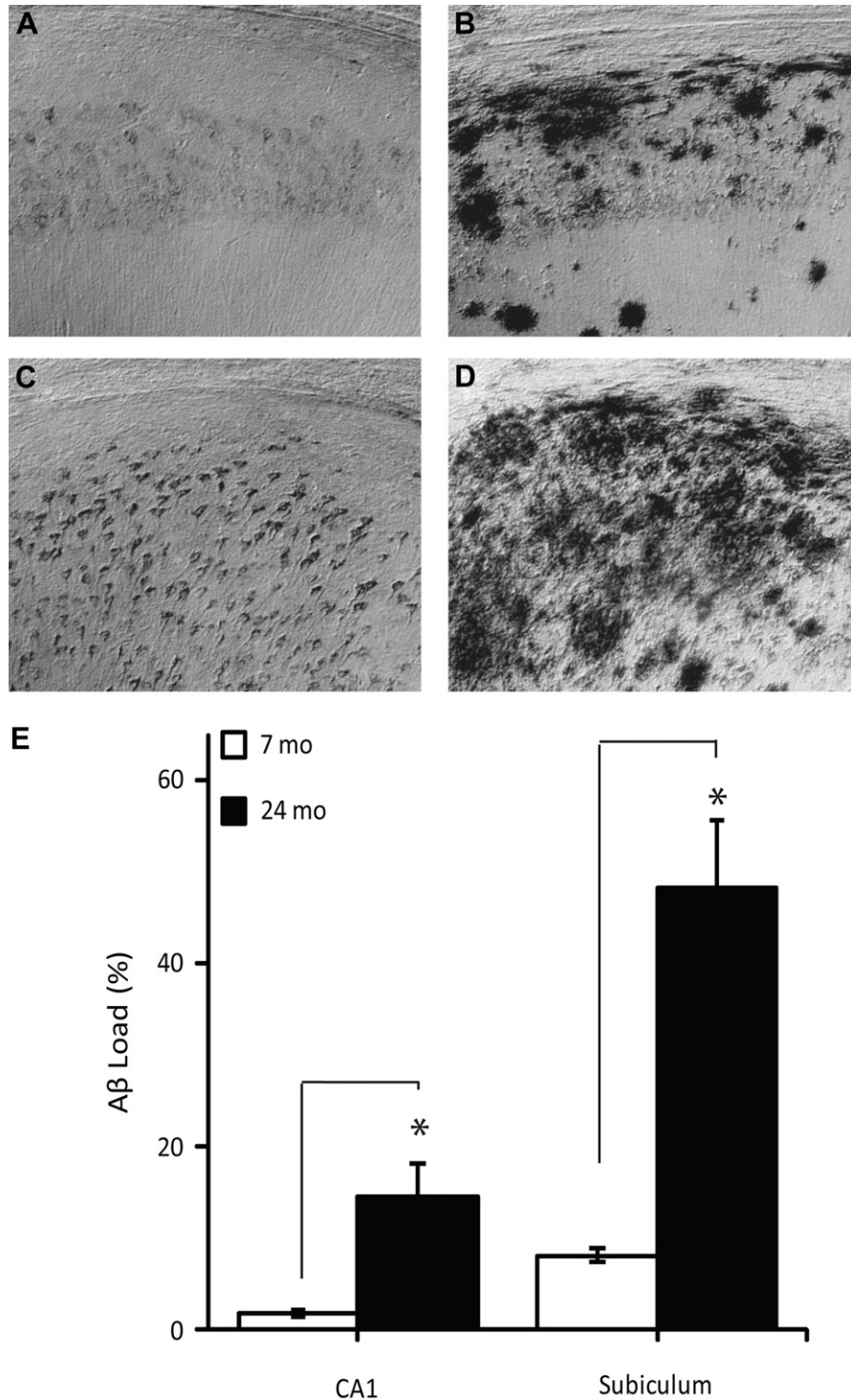
Unpaired Student *t* test was applied to couples of independent variables. Data from experiments with more than 2 groups were analyzed by 2-way analysis of variance, with sex and genotype as 2 independent variables, followed by the Bonferroni posttest. All analyses were performed using GraphPad PRISM (version 5).

### 3. Results

#### 3.1. Development of neuropathology in male 3xTg-AD mice

A $\beta$  immunoreactivity was not detectable in the hippocampal formation of WT mice. Therefore, A $\beta$  immunoreactivity was quantitatively assessed in 3xTg-AD mice only (Fig. 1). A $\beta$  immunoreactivity

was most prominent in the subiculum region of both young and aged mice (Fig. 1C and D). In the hippocampus of the young adult mice, A $\beta$  immunoreactivity was restricted to the CA1 region, primarily observed in the pyramidal cell layer (Fig. 1A). In the aged mice, hippocampal A $\beta$  immunoreactivity was observed throughout CA1–3 regions and the dentate gyrus (Fig. 1B). In the aged mice, A $\beta$  immunoreactive load was greater than 5-fold higher in the hippocampus



**Fig. 1.** Age-related increase in  $\beta$ -amyloid (A $\beta$ ) immunoreactivity in 3xTg-AD mice. Representative photomicrographs show A $\beta$  immunoreactivity in hippocampus CA1 (A, B) and subiculum (C, D) from 7-month-old (A, C) and 24-month-old (B, D) 3xTg-AD mice. (E) A $\beta$  immunoreactive load values in the hippocampus CA1 and subiculum of 7-month-old ( $n = 7$ ) and 24-month-old ( $n = 7$ ) 3xTg-AD mice. \*  $p < 0.005$ .



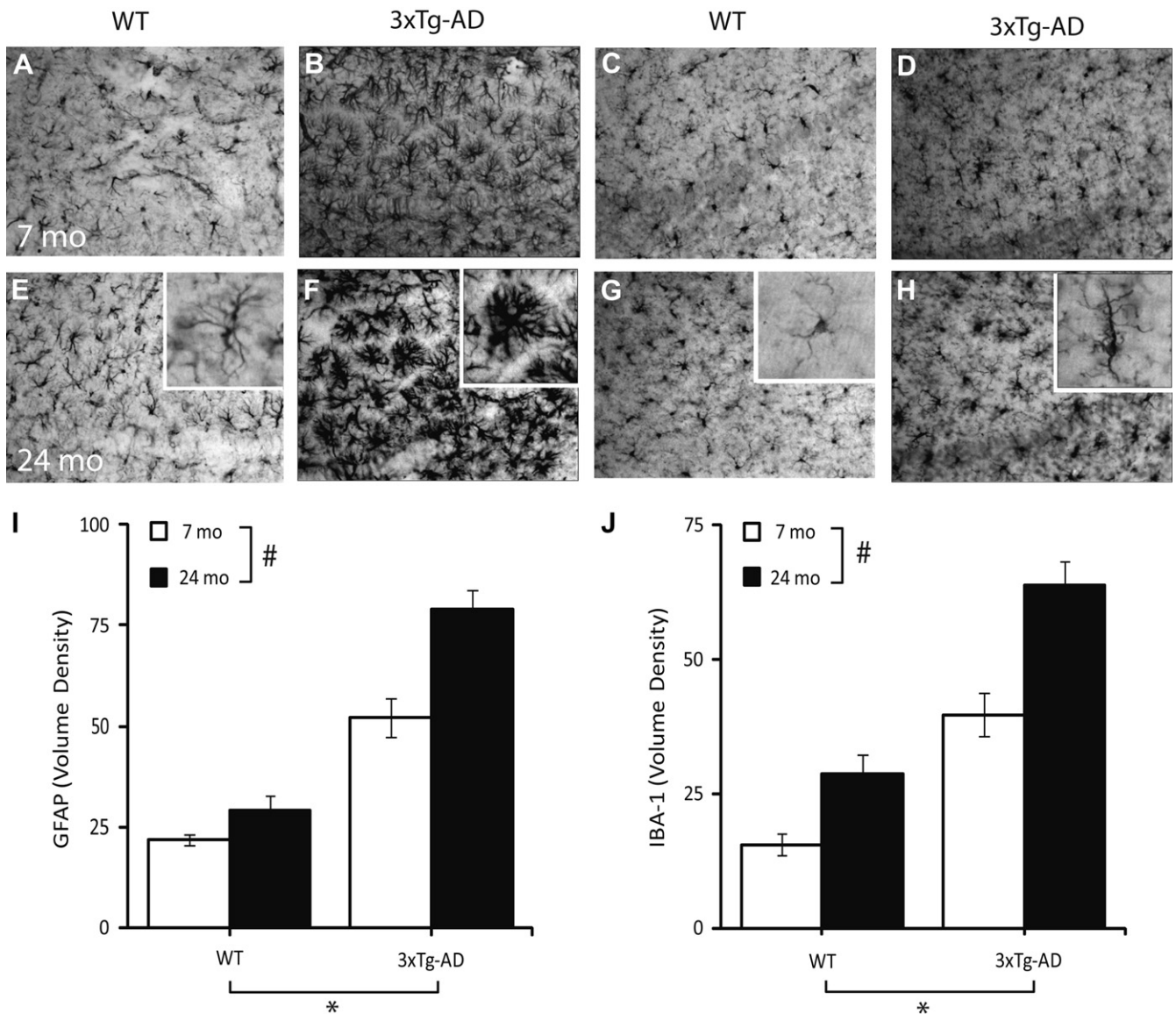
CA1 ( $p < 0.005$ ) and subiculum ( $p < 0.001$ ) regions compared with young adult 3xTg-AD mice (Fig. 1E).

GFAP and IBA-1 immunoreactivities were assessed as markers of gliosis in the hippocampus CA1 (Fig. 2). A significant effect of genotype was observed on both GFAP ( $p < 0.001$ ) and IBA-1 immunoreactivities ( $p < 0.001$ ), with greater than 2-fold higher levels of GFAP and IBA-1 in young adult and aged 3xTg-AD mice compared with WT mice of the same age. A significant effect of age was also observed on GFAP ( $p < 0.001$ ) and IBA-1 ( $p < 0.001$ ) immunoreactivities, with an age-related increase in the markers of gliosis observed in both WT and 3xTg-AD mice at 24 months of age. No significant interaction was observed between genotype and age.

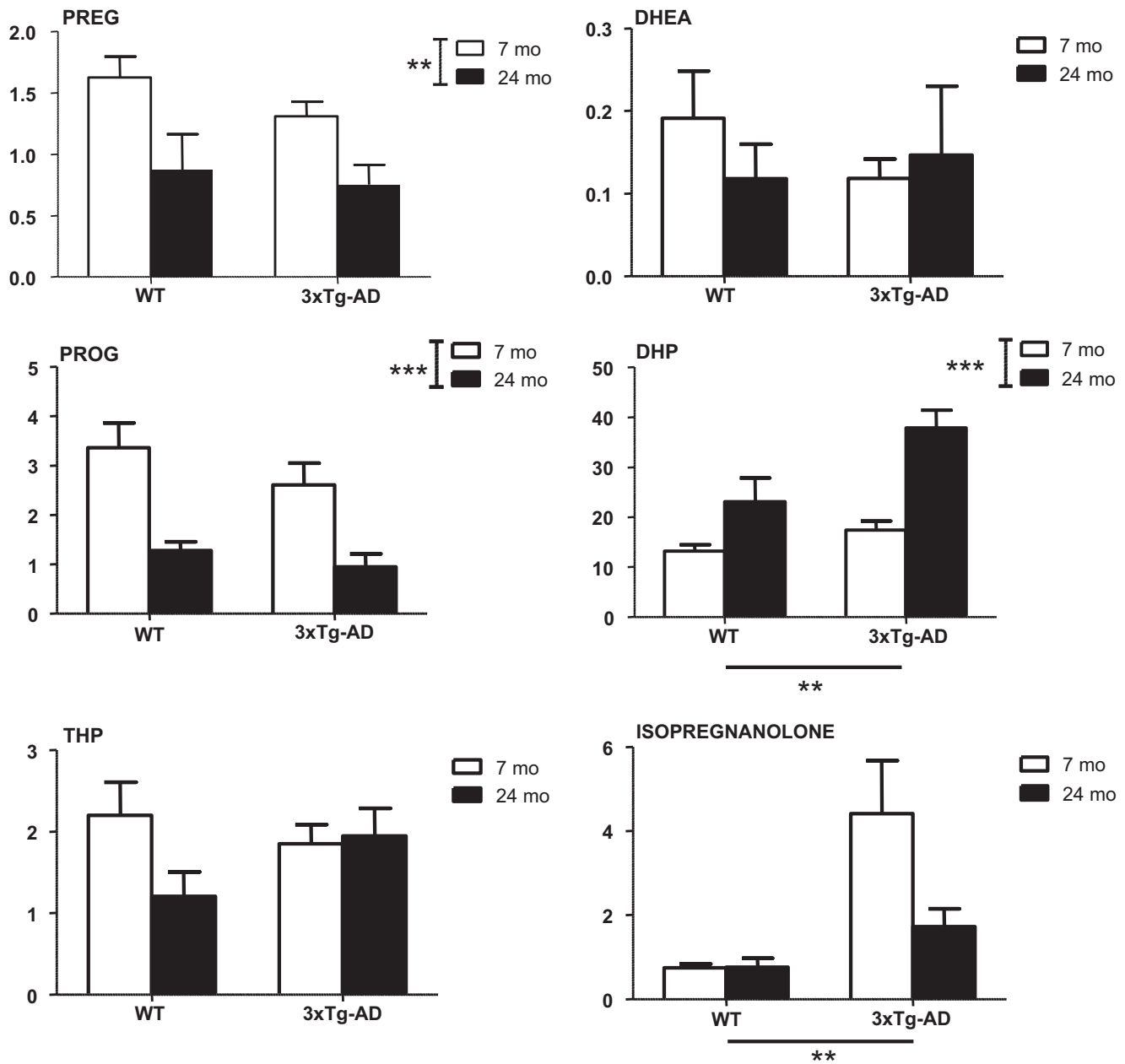
### 3.2. Neuroactive steroid levels in the limbic region of male 3xTg-AD mice

Levels of neuroactive steroids were measured by LC-MS/MS in the limbic region of young adult and aged senescent male

3xTg-AD mice. Levels were compared with those measured in WT animals. A significant effect of genotype ( $p < 0.01$ ) was observed on two PROG metabolites—DHP and isopregnanolone (Fig. 3), and on  $17\beta$ -E (Fig. 4). Increased levels of DHP, isopregnanolone, and  $17\beta$ -E were observed in both young adult and aged 3xTg-AD mice compared with WT mice of the same age (Figs. 3 and 4). In case of DHP, a significant effect of age ( $p < 0.001$ ) also occurred (Fig. 3), with an age-related increase in DHP levels evident in both WT and 3xTg-AD mice at 24 months of age. However, no significant interaction was observed between genotype and age. Age also significantly affected levels of the precursors of DHP, PREG ( $p < 0.01$ ) and PROG ( $p < 0.001$ ), as well as T ( $p < 0.01$ ) and its derivatives, DHT ( $p < 0.01$ ), and  $3\alpha$ -diol ( $p < 0.01$ ) (Figs. 3 and 4). For all these neuroactive steroids, with the exception of  $3\alpha$ -diol, an age-related decrease of their levels occurred in both WT and 3xTg-AD mice at 24 months of age (Figs. 3 and 4). At variance,  $3\alpha$ -diol levels were upregulated with aging in WT and 3xTg-AD mice (Fig. 4).



**Fig. 2.** Age-related increase in markers of gliosis in wild type (WT) and 3xTg-AD mice. Representative photomicrographs show glial fibrillary acidic protein (GFAP) (A, B, E, F) and ionized calcium binding adaptor molecule-1 (IBA-1) (C, D, G, H) immunoreactivity in hippocampus CA1 of 7-month-old WT (A, C), 7-month-old 3xTg-AD (B, D), 24-month-old WT (E, G), and 24-month-old 3xTg-AD mice (F, H). (I) GFAP immunoreactivity volume density values in the CA1 region. (J) IBA-1 immunoreactivity volume density values in the CA1 region. WT, 7-month-old,  $n = 6$ ; WT, 24-month-old,  $n = 5$ ; 3xTg-AD, 7-month-old,  $n = 7$ ; 3xTg-AD, 24-month-old,  $n = 7$ . \*  $p < 0.001$ .

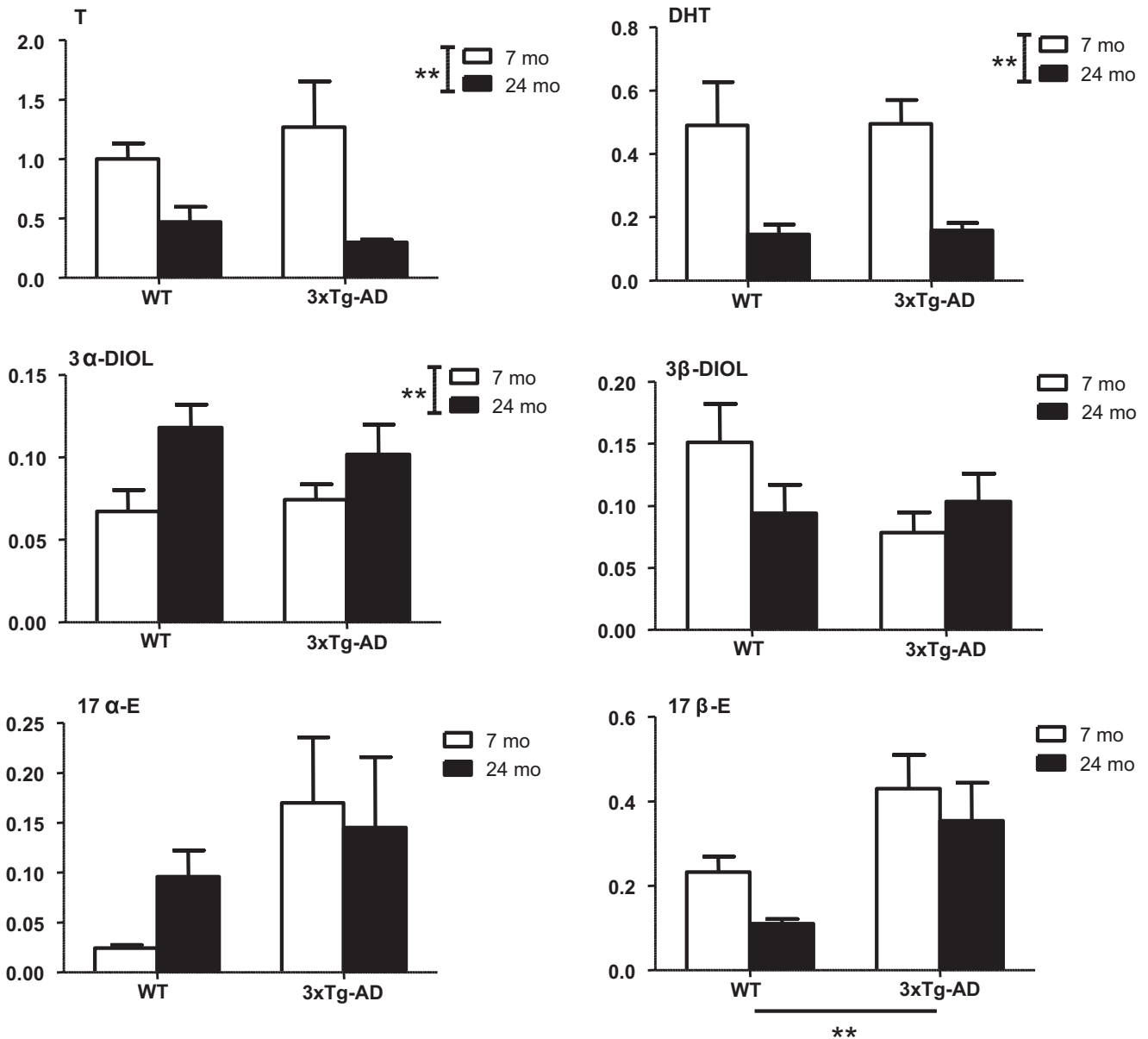


**Fig. 3.** Levels of pregnenolone (PREG), dehydroepiandrosterone (DHEA), progesterone (PROG) and its metabolites, dihydroprogesterone (DHP), tetrahydroprogesterone (THP), and isopregnanolone in the limbic region of male wild type (WT) and 3xTg-AD mice at 7 and 24 months. Data are expressed as pg/mg tissue  $\pm$  standard error of the mean. The effects of age and genotype and the interaction age by genotype have been analyzed using the 2-way analysis of variance test. WT, 7-month-old,  $n = 8$ ; WT, 24-month-old,  $n = 5$ ; 3xTg-AD, 7-month-old,  $n = 8$ ; 3xTg-AD, 24-month-old,  $n = 5$ . \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

To investigate potential relationships between precursors and metabolites we performed correlations between neuroactive steroid levels. In particular, on the basis of the classic steroidogenic pathways shown in Fig. 5, we considered correlations among PREG, PROG and its metabolites (i.e., DHP, THP, and isopregnanolone) in Table 1; and correlations among PREG, DHEA, T, and its metabolites (i.e., DHT, 3 $\alpha$ -diol, and 3 $\beta$ -diol) and estrogens (i.e., 17 $\alpha$ -E and 17 $\beta$ -E) in Table 2. As reported in Table 1, a positive correlation was observed between PREG and DHP in young WT animals ( $p < 0.01$ ). No significant correlation was observed between PREG and DHP in 3xTg-AD mice of the same age or with either aging WT or 3xTg-AD mice. Levels of DHP and THP were positively correlated in young 3xTg-AD mice ( $p < 0.05$ ), and the levels of DHP and

isopregnanolone were negatively correlated in aged 3xTg-AD mice ( $p < 0.01$ ). The levels of THP and isopregnanolone were positively correlated in young and aged WT ( $p < 0.05$ ). This did not occur in 3xTg-AD mice.

As reported in Table 2, PREG levels positively correlated with T ( $p < 0.01$ ), 3 $\alpha$ -diol, 3 $\beta$ -diol, and 17 $\beta$ -E ( $p < 0.05$ ) levels in young WT animals. This correlation was lost in 3xTg-AD mice of the same age and with aging in both WT and 3xTg-AD mice. In young WT animals, DHEA levels were positively correlated with the levels of 3 $\alpha$ -diol, 3 $\beta$ -diol, and 17 $\alpha$ -E ( $p < 0.05$ ). In 3xTg-AD aged animals, DHEA levels were positively correlated with the levels of T, 17 $\alpha$ -E, and 17 $\beta$ -E ( $p < 0.05$ ). T levels in young WT mice were positively correlated with levels of 3 $\beta$ -diol ( $p < 0.05$ ) and of 17 $\beta$ -E ( $p < 0.01$ ).



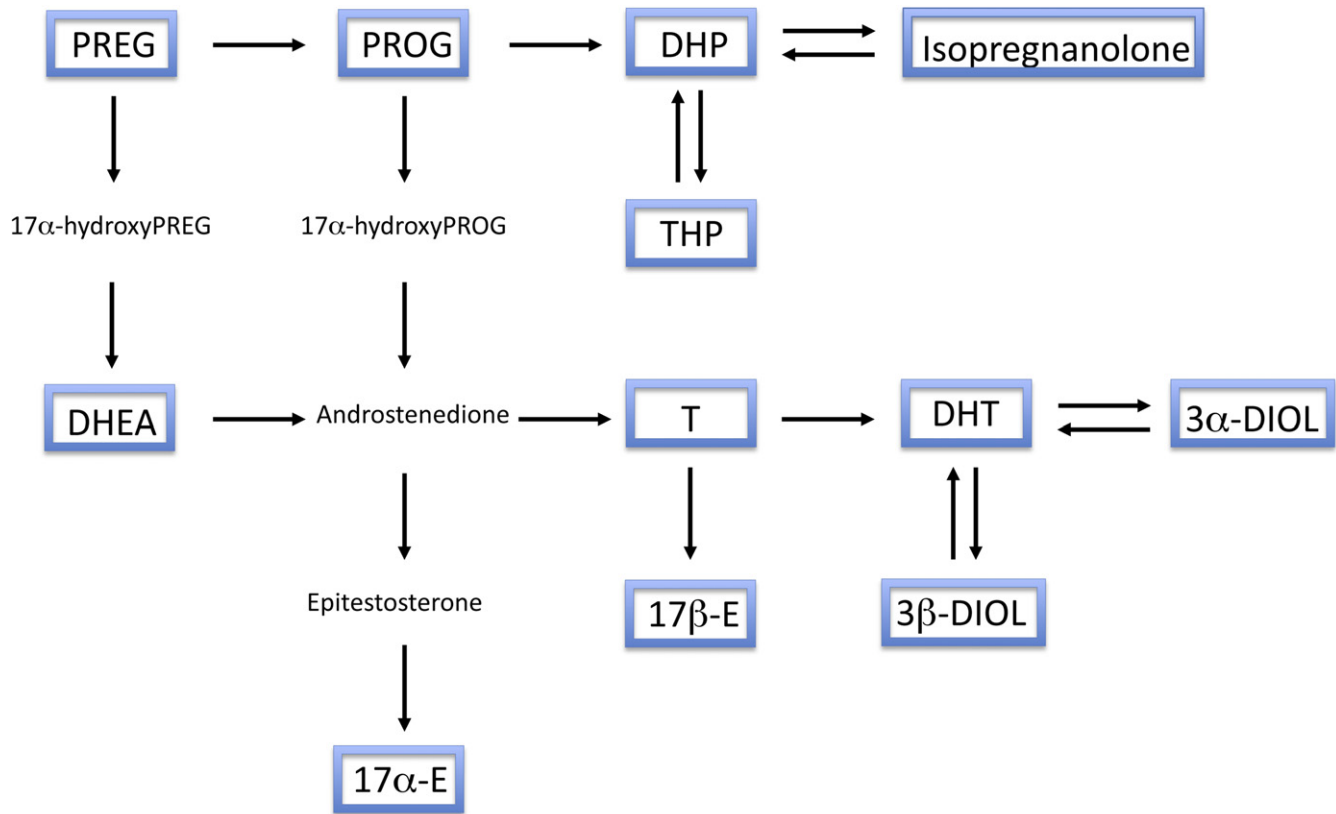
**Fig. 4.** Levels of testosterone (T) and its metabolites, dihydrotestosterone (DHT), 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (3 $\alpha$ -DIOL), 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol (3 $\beta$ -DIOL), 17 $\alpha$ -estradiol (17 $\alpha$ -E), and 17 $\beta$ -estradiol (17 $\beta$ -E) in the limbic region of male wild type (WT) and 3xTg-AD mice at 7 and 24 months. Data are expressed as pg/mg tissue  $\pm$  standard error of the mean. The effects of age and genotype and the interaction age by genotype have been analyzed using the 2-way analysis of variance test. WT, 7-month-old,  $n = 8$ ; WT, 24-month-old,  $n = 5$ ; 3xTg-AD, 7-month-old,  $n = 8$ ; 3xTg-AD, 24-month-old,  $n = 5$ . \*\*  $p < 0.01$ .

In aged WT animals, the levels of T and DHT were positively correlated ( $p < 0.001$ ), and in aged 3xTg-AD mice, the levels of T, 17 $\alpha$ -E, and 17 $\beta$ -E showed a positive correlation ( $p < 0.05$ ). In young WT animals, the levels of the first metabolite of T, DHT, were significantly correlated with those of 3 $\beta$ -diol ( $p < 0.05$ ), and the levels of the other metabolite of DHT, 3 $\alpha$ -diol, were positively correlated with those of 17 $\alpha$ -E ( $p < 0.001$ ). These correlations were lost in 3xTg-AD mice of the same age and with aging in both WT and 3xTg-AD mice. The levels of 3 $\beta$ -diol showed a positive correlation with the levels of 17 $\alpha$ -E ( $p < 0.01$ ) and 17 $\beta$ -E ( $p < 0.05$ ) in young WT animals. 3 $\beta$ -diol levels maintained a positive correlation with the levels of 17 $\beta$ -E ( $p < 0.05$ ) in young 3xTg-AD mice. The levels of 17 $\alpha$ -E and 17 $\beta$ -E showed a positive correlation ( $p < 0.05$ ) in aged 3xTg-AD mice.

#### 4. Discussion

Here we have characterized the effect of normal and pathologic aging on neuroactive steroid levels in the learning and memory center of WT and 3xTg-AD mice. Our findings demonstrate that aging and AD-related neuropathology have important effects on neuroactive steroid homeostasis in the region of the brain most severely affected in AD, the limbic region. The observed effects of age and AD-neuropathology on neuroactive steroid levels might contribute to age-related susceptibility to AD pathogenesis and age-dependent cognitive decline.

For this study we have selected male mice of 7 and 24 months of age, because the degree of pathologic alteration is known to be affected by age in 3xTg-AD mice (Bittner et al., 2010; Caccamo et al., 2010;



**Fig. 5.** Schematic representation of neurosteroidogenesis. Framed neuroactive steroids reported have been assessed by liquid chromatography tandem mass spectrometry. Abbreviations: 17 $\alpha$ -E, 17 $\alpha$ -estradiol; 17 $\beta$ -E, 17 $\beta$ -estradiol; 3 $\alpha$ -DIOL, 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol; 3 $\beta$ -DIOL, 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol; DHEA, dehydroepiandrosterone; DHP, dihydroprogesterone; DHT, dihydrotestosterone; PREG, pregnenolone; PROG, progesterone; T, testosterone; THP, tetrahydroprogesterone.

Ghosh et al., 2012; Olabarria et al., 2011; Overk et al., 2009). In fact, the pathologic examination of the brain of 3xTg-AD mice revealed a 5-fold higher A $\beta$  immunoreactive load in CA1 and subiculum at 24 months of age in comparison with 7 months of age. The volume density of GFAP immunoreactive astrocytes and IBA-1 immunoreactive microglia was also increased at 24 months of age versus 7 months of age in both 3xTg-AD and WT mice. Furthermore, the volume density of GFAP immunoreactive astrocytes and IBA-1 immunoreactive microglia was increased in 3xTg-AD mice compared with WT at both ages. Therefore, A $\beta$  immunoreactive load and gliosis were affected by age and genotype. Interestingly,

neuroactive steroid levels, analyzed by LC-MS/MS in the limbic region, were also affected by age and genotype.

Our data extend previous observations showing that neuroactive steroid levels are affected by aging in the central nervous system of nonpathologic animals (Schumacher et al., 2003). We observed an age-related decrease in the levels of PROG, together with an increase in the levels of its metabolite DHP, in the limbic region of WT and 3xTg-AD male mice. The levels of other neuroactive steroids were also affected by aging. For instance, in both WT and 3xTg-AD aged mice, the levels of T and its direct metabolite, DHT, were both decreased. Previous studies have similarly reported

**Table 1**  
Correlation (*r* value) between the levels of pregnenolone, progesterone, and its metabolites in limbic area of young (7 months) and aged (24 months) WT or TG rats

|                       | PREG              |    | PROG |    | DHP |                    | THP               |    | Isopregnanolone |    |
|-----------------------|-------------------|----|------|----|-----|--------------------|-------------------|----|-----------------|----|
|                       | WT                | TG | WT   | TG | WT  | TG                 | WT                | TG | WT              | TG |
| Young ( <i>n</i> = 8) |                   |    |      |    |     |                    |                   |    |                 |    |
| PREG                  | -                 | -  |      |    |     |                    |                   |    |                 |    |
| PROG                  | NS                | NS | -    | -  |     |                    |                   |    |                 |    |
| DHP                   | 0.75 <sup>a</sup> | NS | NS   | NS | -   | NS                 |                   |    |                 |    |
| THP                   | NS                | NS | NS   | NS | NS  | 0.66 <sup>b</sup>  |                   |    |                 |    |
| Isopregnanolone       | NS                | NS | NS   | NS | NS  | NS                 | 0.50 <sup>b</sup> | -  | NS              | -  |
| Aged ( <i>n</i> = 5)  |                   |    |      |    |     |                    |                   |    |                 |    |
| PREG                  | -                 | -  |      |    |     |                    |                   |    |                 |    |
| PROG                  | NS                | NS | -    | -  |     |                    |                   |    |                 |    |
| DHP                   | NS                | NS | NS   | NS | -   | -                  |                   |    |                 |    |
| THP                   | NS                | NS | NS   | NS | NS  | NS                 |                   |    |                 |    |
| Isopregnanolone       | NS                | NS | NS   | NS | NS  | -0.87 <sup>a</sup> | 0.76 <sup>b</sup> | -  | NS              | -  |

Key: DHP, dihydroprogesterone; NS, not significant; PREG, pregnenolone; PROG, progesterone; TG, 3xTg-AD; THP, tetrahydroprogesterone; WT, wild type.

<sup>a</sup> *p* < 0.01.

<sup>b</sup> *p* < 0.05.

**Table 2**Correlation (*r* value) between the levels of pregnenolone, dehydroepiandrosterone, testosterone and its metabolites, and estrogens in limbic area of young (7 months) and aged (24 months) WT or TG mice

|                       | PREG              |    | DHEA              |                   | T                 |                   | DHT               |    | 3 $\alpha$ -DIOL |                   | 3 $\beta$ -DIOL |                   | 17 $\alpha$ -E    |    | 17 $\beta$ -E     |    |   |
|-----------------------|-------------------|----|-------------------|-------------------|-------------------|-------------------|-------------------|----|------------------|-------------------|-----------------|-------------------|-------------------|----|-------------------|----|---|
|                       | WT                | TG | WT                | TG                | WT                | TG                | WT                | TG | WT               | TG                | WT              | TG                | WT                | TG | WT                | TG |   |
| Young ( <i>n</i> = 8) |                   |    |                   |                   |                   |                   |                   |    |                  |                   |                 |                   |                   |    |                   |    |   |
| PREG                  | -                 | -  |                   |                   |                   |                   |                   |    |                  |                   |                 |                   |                   |    |                   |    |   |
| DHEA                  | NS                | NS | -                 | -                 |                   |                   |                   |    |                  |                   |                 |                   |                   |    |                   |    |   |
| T                     | 0.77 <sup>a</sup> | NS | NS                | NS                | -                 | -                 |                   |    |                  |                   |                 |                   |                   |    |                   |    |   |
| DHT                   | NS                | NS | NS                | NS                | NS                | NS                | -                 | -  |                  |                   |                 |                   |                   |    |                   |    |   |
| 3 $\alpha$ -DIOL      | 0.57 <sup>b</sup> | NS | 0.50 <sup>b</sup> | NS                | NS                | NS                | NS                | NS | -                | -                 |                 |                   |                   |    |                   |    |   |
| 3 $\beta$ -DIOL       | 0.64 <sup>b</sup> | NS | 0.51 <sup>b</sup> | NS                | 0.54 <sup>b</sup> | NS                | 0.55 <sup>b</sup> | NS | 0.55             | NS                | -               | -                 |                   |    |                   |    |   |
| 17 $\alpha$ -E        | NS                | NS | 0.63 <sup>b</sup> | NS                | NS                | NS                | NS                | NS | NS               | 0.89 <sup>c</sup> | NS              | 0.75 <sup>a</sup> | NS                | -  | -                 |    |   |
| 17 $\beta$ -E         | 0.60 <sup>b</sup> | NS | NS                | NS                | 0.73 <sup>a</sup> | NS                | NS                | NS | NS               | NS                | NS              | 0.56 <sup>b</sup> | 0.66 <sup>b</sup> | NS | NS                | -  | - |
| Aged ( <i>n</i> = 5)  |                   |    |                   |                   |                   |                   |                   |    |                  |                   |                 |                   |                   |    |                   |    |   |
| PREG                  | -                 | -  |                   |                   |                   |                   |                   |    |                  |                   |                 |                   |                   |    |                   |    |   |
| DHEA                  | NS                | NS | -                 | -                 |                   |                   |                   |    |                  |                   |                 |                   |                   |    |                   |    |   |
| T                     | NS                | NS | NS                | 0.75 <sup>b</sup> | -                 | -                 |                   |    |                  |                   |                 |                   |                   |    |                   |    |   |
| DHT                   | NS                | NS | NS                | NS                | 0.99 <sup>c</sup> | NS                | -                 | -  |                  |                   |                 |                   |                   |    |                   |    |   |
| 3 $\alpha$ -DIOL      | NS                | NS | NS                | NS                | NS                | NS                | NS                | NS | -                | -                 |                 |                   |                   |    |                   |    |   |
| 3 $\beta$ -DIOL       | NS                | NS | NS                | NS                | NS                | NS                | NS                | NS | NS               | NS                | -               | -                 |                   |    |                   |    |   |
| 17 $\alpha$ -E        | NS                | NS | NS                | 0.89 <sup>a</sup> | NS                | 0.84 <sup>b</sup> | NS                | NS | NS               | NS                | NS              | NS                | NS                | -  | -                 |    |   |
| 17 $\beta$ -E         | NS                | NS | NS                | 0.74 <sup>b</sup> | NS                | 0.74 <sup>b</sup> | NS                | NS | NS               | NS                | NS              | NS                | NS                | NS | 0.90 <sup>a</sup> | -  | - |

Key: 17 $\alpha$ -E, 17 $\alpha$ -estradiol; 17 $\beta$ -E, 17 $\beta$ -estradiol; 3 $\alpha$ -DIOL, 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol; 3 $\beta$ -DIOL, 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol; DHEA, dehydroepiandrosterone; DHT, dihydrotestosterone; NS, not significant; PREG, pregnenolone; T, testosterone; TG, 3 $\alpha$ Tg-AD; WT, wild type.

<sup>a</sup> *p* < 0.01.

<sup>b</sup> *p* < 0.05.

<sup>c</sup> *p* < 0.001.

an age-related depletion in androgens in male rats (Rosario et al., 2009) and human frontal cortex (Rosario et al., 2011). Interestingly, in men, circulating levels of DHT do not change with age (Kaufman and Vermeulen, 2005), despite depleted brain levels of DHT (Rosario et al., 2011), highlighting the importance of characterizing age-related neuroactive steroid changes directly in the brain.

These age-related changes in neuroactive steroid levels might contribute to susceptibility to cognitive decline and AD-related neuropathology with advancing age. Furthermore, previous studies have demonstrated that androgens elicit anti-inflammatory effects, for example testosterone reduces reactive gliosis after a stab wound in castrated rats (Arevalo et al., 2010; Barreto et al., 2007). Therefore, the age-related decline in androgens might contribute to the increased levels of gliosis observed in aged mice. Indeed, age-related depletion of androgens in males is believed to be an important risk factor for AD and might promote A $\beta$  accumulation, with previous studies demonstrating that testosterone exerts a therapeutic effect in 3 $\alpha$ Tg-AD mice (Rosario et al., 2010).

The decrease in the levels of T and its direct metabolite, DHT, was associated with an increase in the levels of the further metabolite, 3 $\alpha$ -diol. It is interesting to note that at variance to T and DHT, which bind to androgen receptor, 3 $\alpha$ -diol interacts with GABA-A receptor (Melcangi et al., 2008). Thus, the increase in 3 $\alpha$ -diol levels with aging might affect activation of GABA-A receptor.

In addition to being affected by aging, our findings suggest that AD neuropathology might also alter neuroactive steroid levels. Previous observations in postmortem brain tissue of AD patients have demonstrated altered neuroactive steroid levels in brain regions affected by AD neuropathology (Luchetti et al., 2011a, 2011b; Marx et al., 2006; Rosario et al., 2011; Yue et al., 2005). However, these studies have not been able to clearly determine if altered neuroactive steroid levels are a risk factor for AD or an outcome of AD pathogenesis. In the 3 $\alpha$ Tg-AD mice, an age-dependent increase in A $\beta$  pathology was associated with increased markers of gliosis compared with WT mice. Furthermore, increased levels of the neuroactive steroids DHP, isopregnanolone, and 17 $\beta$ -E were also observed in 3 $\alpha$ Tg-AD compared with WT mice.

In the current study, androgens were affected by aging but not AD neuropathology in the 3 $\alpha$ Tg-AD mice, supporting the notion that in the male brain, depleted androgens is a risk factor for AD, not a consequence of AD neuropathology.

Concerning PROG metabolites in the limbic region, we detected a significant increase in the levels of DHP and isopregnanolone, in 3 $\alpha$ Tg-AD compared with WT mice. In addition, DHP positively correlated with THP in young 3 $\alpha$ Tg-AD mice, and negatively correlated isopregnanolone in aged 3 $\alpha$ Tg-AD mice. These correlations were not detected in WT mice. In contrast, 3 $\alpha$ Tg-AD mice did not show the positive correlations in the levels of PREG and DHP and in the levels of THP and isopregnanolone that were detected in young and older WT mice, respectively. These findings suggest that the metabolism of PROG is altered in the limbic region of 3 $\alpha$ Tg-AD mice. This alteration is of relevance, because PROG metabolites have been shown to exert neuroprotective actions (Brinton and Wang, 2006; Brinton et al., 2008; Sun et al., 2012). Indeed, treatment of 3 $\alpha$ Tg-AD mice with THP decreases A $\beta$  accumulation (Chen et al., 2011), reverses cognitive deficits, and promotes neurogenesis (Singh et al., 2011; Wang et al., 2010). The protective action of THP might be related with its ability to activate the GABA-A receptor (Melcangi et al., 2008). In contrast, isopregnanolone, which as shown here is increased in the limbic region of 3 $\alpha$ Tg-AD mice, does not bind directly to the GABA-A receptor (Bitran et al., 1991), but it antagonizes the effect of THP on the GABA-A receptor (Wang et al., 2002). Moreover, in this context, it is also important to highlight that, as recently reported (Luchetti et al., 2011a, 2011b), the expression of several GABA-A receptor subunits is significantly reduced in postmortem brain tissue of AD patients. Indeed, alterations of GABA signals might play an important role in the cognitive and behavioral alterations which occur in AD (Birzniece et al., 2006; Lanctot et al., 2007). Therefore, brain alterations in PROG metabolites that modulate GABA-A receptor function, might contribute to the cognitive alterations of AD patients.

Another important modification detected in the limbic region of 3 $\alpha$ Tg-AD mice was a significant increase in the levels of 17 $\beta$ -E. This might represent an endogenous neuroprotective response to A $\beta$  accumulation in the 3 $\alpha$ Tg-AD mice. Indeed, aromatase, the enzyme



responsible for the synthesis of 17 $\beta$ -E from T, shows increased expression under neurodegenerative conditions (Azcoitia et al., 2001; Garcia-Segura and Balthazart, 2009; Garcia-Segura et al., 2003), including within the AD brain (Luchetti et al., 2011a, 2011b). In the aged 3xTg-AD mice, DHEA and T levels were positively correlated with 17 $\beta$ -E levels, supporting the notion that the elevated 17 $\beta$ -E levels observed in the 3xTg-AD mice are endogenously synthesized in the limbic region from T. Furthermore, 3 $\beta$ -diol levels showed a positive correlation with 17 $\beta$ -E levels in young 3xTg-AD mice. These findings suggest a role in AD pathology for androgen receptor, target of the action of T and possibly of DHEA, and for estrogen receptors, target of the action of 17 $\beta$ -E and 3 $\beta$ -diol. Indeed, androgens and estrogens have been shown to exert a therapeutic effect in 3xTg-AD mice (Carroll et al., 2007; Rosario et al., 2006, 2010).

In conclusion, the present results show age-related modification in specific neuroactive steroid levels in the limbic region of 3xTg-AD mice brains in association with age-related pathologic changes (i.e., A $\beta$  immunoreactive load and gliosis). Because of the well-ascertained neuroprotective capacity of neuroactive steroids, the present findings might represent a preclinical background for a therapy based on these molecules to be applied in AD. In particular, the fact that some neuroactive steroids observed to be modified in 3xTg-AD mice are able to interact with classic steroid receptors (i.e., androgen, progesterone, or estrogen receptors), and others interact with nonclassic steroid receptors (i.e., GABA-A receptor), might open new therapeutic strategies based on specific synthetic ligands for classic and nonclassic steroid receptors.

## Disclosure statement

There are no actual or potential conflicts of interest.

All experimentation was approved by the USC Institutional Animal Care and Use Committee and carried out in accordance with National Institutes of Health guidelines.

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